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POTENTIAL NOVEL MOLECULAR TARGETS FOR BREAST CANCER DIAGNOSIS AND TREATMENT

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POTENTIAL NOVEL MOLECULAR TARGETS FOR BREAST CANCER DIAGNOSIS AND TREATMENT

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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In the name of Allah, Most Gracious, Most Merciful

To my wife, Zahra, and my daughters, Fatemeh and Maryam

ABSTRACT

Breast cancer possesses the highest incidence among cancers in women worldwide, accounting for approximately 26% of all cancers in women. Due to the discovery of specific predictive and prognostic biomarkers, breast cancer therapy has been significantly promoted in the past decades through the application of more individualized therapies to diverse subcategories with different clinical behavior. Among the established biomarkers for breast cancer, estrogen receptor alpha (ERα) and human epidermal growth factor receptor 2 (HER2) are the most potent biomarkers both in determining prognosis and predicting response to hormone therapies. However, there is a clear need to identify additional biomarkers as some subtypes of breast cancer do not express ERα and/or HER2 and additionally, there is no perfect correlation between these biomarkers and the response to targeted treatment. Therefore, the overall aim of this thesis was to evaluate potential new biomarkers for breast cancer diagnosis and to improve therapeutic strategies in breast cancer.

Estrogen signaling has been proven to play a key role in breast cell growth, differentiation and development of breast cancer. It has been well documented that ERα can directly interact with *cis*-regulatory elements; estrogen response elements (EREs) or indirectly with other *cis*-regulatory elements via protein-protein interactions, such as activator protein-1 (AP-1), to regulate transcription of target genes. The AP-1 as a transcription factor is a dimeric complex that includes members of the JUN and FOS protein families. Studies have indicated a role for these proteins as potential biomarkers in breast cancer. However, a systemic analysis of the expression of all AP-1 family members as potential biomarkers in breast cancer and their interaction with ERα is still lacking.

In **paper I**, we examined the expression levels of seven AP-1 family members in human breast tumors and adjacent non-tumor tissues and correlated their expression with available clinicopathological parameters. We observed that the expression of all AP-1 family members except Fos-B was significantly elevated in tumor compared with adjacent non-tumor tissues. Interestingly, we observed that the Fra-1 expression level was significantly higher in the tumors classified as ERα-negative and progesterone receptor (PR) negative. Furthermore, Fra-1 expression was shown to significantly distinguish triple-negative tumors compared from luminal carcinomas.

ERα is overexpressed in the majority of breast cancers and promotes estrogen-dependent cancer progression by regulating the transcription of genes related to cell proliferation. Anti-estrogens are successfully used to treat these tumors. However, in many cases resistance to

this endocrine treatment develops. Therefore, insights into the molecular mechanisms that regulate ERα expression and stability are of highest importance to promote breast cancer diagnostics and therapeutics.

In **paper II**, we found that the atypical E3 ubiquitin ligase RNF31 can stabilize ERα and facilitate ERα-stimulated proliferation in breast cancer cell lines. This study proposes a non-genomic mechanism by which RNF31 regulates ERα expression and stability and controls the transcription of estrogen-dependent genes related to breast cancer cell proliferation.

RNF31 is one of three members in the linear ubiquitin chain assembly complex (LUBAC). In **paper III**, we investigated mRNA and protein expression levels of all three members of the LUBAC complex, including RBCK1, RNF31 and SHARPIN, in human breast tumors and adjacent non-tumor tissues and correlated their expression with various clinicopathological parameters. We found that all members of the LUBAC complex were significantly higher expressed in tumors compared to adjacent non-tumor tissues. We also found that the RNF31 protein expression level was significantly higher in ERα-negative tumors compared to ERα-positive tumors.

In **paper IV**, we identified the existence of a potential fusion transcript, called RNF31/IRF9, and a corresponding potential fusion protein. Interestingly, the potential novel fusion protein was present in the nuclei of breast tumors but not in the nuclei of normal breast tissues. In addition, the expression of the potential fusion protein was significantly higher in ERα-positive tumors compared to ERα-negative tumors.

In summary, the work presented in this thesis contribute to the understanding of estrogen signaling in breast cancer and identify and suggest a group of proteins that are candidates as potential novel biomarkers and/or drug targets to improve therapeutic strategies in breast cancer.

LIST OF SCIENTIFIC PAPERS

I. **Kharman-Biz A**, Gao H, Ghiasvand R, Zhao C, Zendehdel K, Dahlman-Wright K. *Expression of activator protein-1 (AP-1) family members in breast cancer.* BMC cancer (2013) 13: 441.

II. Zhu J, Zhao C, **Kharman-Biz A**, Zhuang T, Jonsson P, Zhuang T, Jonsson P, Liang N, Williams C, Lin C-Y, Qiao Y, Zendehdel K, Strömblad, Treuter E and Dahlman-Wright K. *The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor alpha and modulates estrogen-stimulated breast cancer cell proliferation.* Oncogene (2014) 33, 4340–4351.

III. **Kharman-Biz A**, Gao H, Ghiasvand R, Haldosen LA, Zendehdel K and Dahlman-Wright K. *Expression of three members of the linear ubiquitin assembly complex in breast cancer.* Manuscript

IV. **Kharman-Biz A**, Gao H, Ghiasvand R, Kuiper R, Haldosen LA, Zendehdel K, Dahlman-Wright K. *Identification of a Potential Novel Fusion Transcript, RNF31/IRF9, in Breast Cancer.* Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AI	Aromatase inhibitor
AJCC	American Joint Committee on Cancer
AP-1	Activator protein-1
ATF	Activating transcription Factor
AUC	Area under the curve
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
bZIP	Basic leucine zipper
ChIP	Chromatin immunoprecipitation
ChIP-SEQ	Chromatin immunoprecipitation followed by sequencing
CML	Chronic myeloid leukemia
CRE	Cyclic AMP-response element
DBD	DNA binding domain
E2	17β-estradiol
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
ERE	Estrogen response element
GPCR	G protein complex receptor
HER2	Human epidermal growth factor receptor 2
IFA	Immunofluorescent assay
IHC	Immunohistochemistry
IKK	IkB kinase
LBD	Ligand binding domain
Ln	Natural logarithm
LUBAC	Linear ubiquitin chain assembly complex
MAF	Musculoaponeurotic fibrosarcoma
MMP	Matrix Metalloproteinase
mRNA	Messenger ribonucleic acid

MTA	Material transfer agreement
NFkB	Nuclear factor kappa beta
PR	Progesterone receptor
PTM	Posttranslational modification
RACE	Rapid Amplification of cDNA Ends
RBCK1	RanBP-type and C3Hc4-type zinc finger containing 1
RBR	Ring between rings
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNF31	RING (Really Interesting New Gene) finger 31
ROC	Receiving operating characteristics
SERMs	Selective estrogen receptor modulators
SHARPIN	SHANK-associated RH domain interacting protein
siRNA	Small interference RNA
SNP	Single nucleotide polymorphism
SP-1	Specific protein-1
TAD	Trans activating domain
TF	Transcription factor
TNBC	Triple negative breast cancer
TNFα	Tumor necrosis factor alpha
TP53	Tumor protein 53
TRE	TPA response element
UBA	Ubiquitin-associated
UBC	Ubiquitin C
UBL	Ubiquitin-like
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 CANCER

Cancer can be described as an abnormal and uncontrolled proliferation of cells. Cancer cells often spread into the surrounding tissue or metastasize to distant organs through the blood or the lymphatic system [1, 2]. Cancer cells can arise in a lot of tissues and organs [3]. Despite advances in early detection and therapy, cancer still is a big health challenge with the highest priority for investigation [4, 5]. As shown in figure 1, the first step of cancer formation is genetic mutation, the "Initiation" phase. "Initiators", which cause or support the process of genetic mutations, include hormones, chemicals, radiation, infection and hypoxia [6, 7]. Genetic mutations can take place in pro-oncogenic genes such as *RAS* [8] and *MYC* [9] or in tumor suppressor genes such as *BRCA1*, *BRCA2* and *TP53* [10, 11]. Generally, cancer development requires the accumulation of multiple genetic aberrations [12, 13]. Mutated cells can stay in a dormant phase or become proliferative. The second step of cancer formation, the "Promotion" phase, includes several steps including hyperplasia (increase in the number of cells), dysplasia (phenotypic changes in cells), *in situ* carcinoma (early stage cancer) and finally invasive carcinoma (spread to the surrounding tissues) [14].

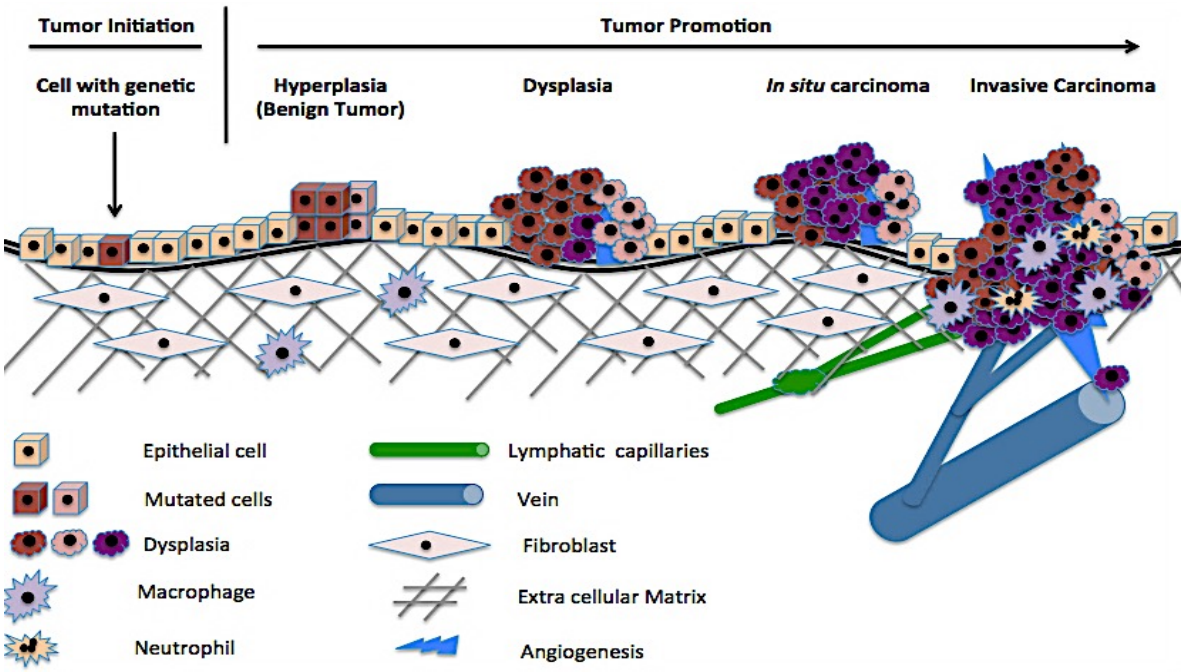

Figure 1. Tumor development steps from normal cells to metastasis. The development of cancer begins when a single mutated cell is initiated to abnormally proliferate. Additional mutations followed by selection of more rapidly proliferating altered cells within the population lead to progression and then invasion to the surrounding connective tissues. The altered cells can spread to distant organs through the blood and lymphatic vessels.

To date, six hallmarks of cancer have been described by which cancer cells sustain their abnormal growth and escape growth suppressor mechanisms [15]. These include sustaining of proliferating signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activated invasion and metastasis [15].

1.2 BREAST CANCER

1.2.1 Definition

Breast cancer is a malignant tumor arising from epithelial cells of glandular milk ducts or lobules of the breast [16]. Breast carcinoma is classified as either non-invasive (carcinoma *in situ*) or invasive, depending on whether or not the tumor has started to grow outside the basal membrane. Invasive carcinomas are cancers in which the altered cells diffuse to surrounding connective tissues and metastasize to distant organs of the body. Around two-thirds of breast carcinomas arise from epithelial cells of the ducts, called ductal carcinoma, and around one-third from lobules, called lobular carcinoma [17]. Other less common histological groups are identified as inflammatory, medullary, apocrine, mucinous and tubular carcinomas as shown in figure 2 [17].

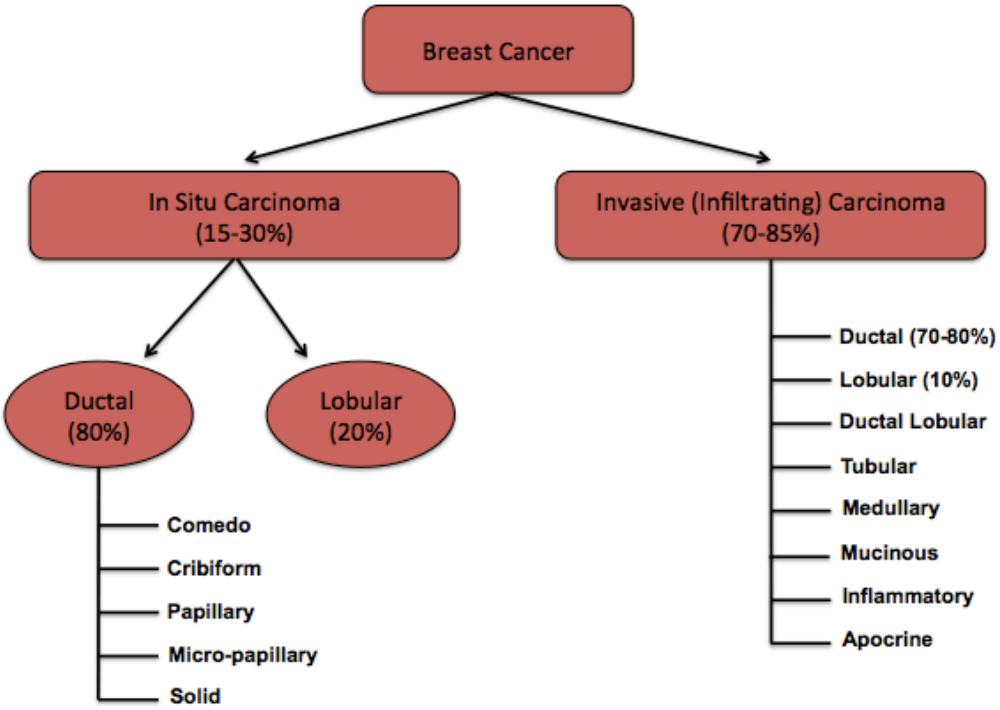

Figure 2. Histological stratification of breast cancer. The majority of breast carcinomas arises from ductal epithelial cells and tends to involve the surrounding connective tissues (invasive ductal carcinoma) and metastasize to the distant organs of the body.

1.2.2 Epidemiology

Breast cancer is the most frequently diagnosed cancer disease in women worldwide, with an estimated one and a half million new cases each year and approximately half a million deaths per year [18]. The incidence rate of breast cancer is steadily increasing worldwide, and vary almost four-fold across world regions, where the rate ranges from 27 per 100,000 in Middle Africa and Eastern Asia to 92 per 100,000 in North America [18]. This can be due to differences in age distribution, diet, lifestyle, ethnicity, genetic background and other breast cancer risk factors between populations.

1.2.3 Treatment

Surgery is introduced to patients having primary breast cancer as the first choice [19]. Breast-conserving surgery is most often chosen, followed by local radiation treatment [20]. This treatment is curative for a large group of patients having breast cancer [21]. Globally, the need for mastectomy (removal of the entire breast) has been significantly decreased due to mammographic screening programs that detect tumors in early stages. To eradicate potential undetectable micro-metastases after surgery, patients often receive adjuvant therapy including chemo, endocrine, and/or targeted therapies. However, depending on the breast cancer subgroup such as inflammatory, some patients may receive neo-adjuvant therapy by which the primary tumor shrinks before surgery [19]. An additional advantage of this treatment is that it provides opportunities to study the tumor response to a therapy.

1.2.4 Biomarkers in breast cancer

Tumor biomarker is defined as a molecule, which is produced by a tumor or in response to a tumor [22, 23]. Biomarkers can be detected from any tissue in the body including breast [22, 23]. They may have prognostic, diagnostic and/or predictive values [24]. Prognostic biomarkers foretell the natural disease course regardless of treatment, while predictive biomarkers foresee the response of a patient to a specific treatment [25]. The expression levels of hormone receptors such as ERα and PR are good examples of weak prognostic but strong predictive biomarkers [26]. Whereas, the overexpression of HER2 could be a proper example of both a strong prognostic biomarker and a strong predictive biomarker [26]. However, in addition to the established biomarkers, a large number of other biomarkers have already been proposed, most of which could not be validated and qualified practically for clinical use. Biomarkers must overcome many practical hurdles and pass five conceptual phases before they are applied in the clinics. These five steps include I) preclinical

exploratory, II) clinical assay and validation, III) retrospective longitudinal, IV) prospective screening, and V) cancer control [27].

1.2.5 Established classifications in breast cancer

To specify the precise prognosis and plan an effective therapy, breast cancer classification is of utmost importance. Therefore, in the following the most established classifications in breast cancer including molecular subtypes of breast cancer, TNM staging system and grade will be discussed.

1.2.5.1 Molecular subtypes of breast cancer

Breast tumors can be classified into four distinct different subtypes using four well-known biomarkers, including ERα, PR, HER2, and Ki-67 (Table 1) [28]. This molecular subtype classification is often key reference for prognosis and choice of therapeutic strategy [29]. Luminal A is the most common subtype that is ERα-positive, PR-positive, HER2-negative and has low expression of Ki-67, and also has the best outcome with hormonal therapy [30]. Luminal B is similar to luminal A but has high expression of Ki-67, which is a proliferation-related gene, and this subtype is more aggressive than luminal A [30, 31]. Patients having luminal B subtype can benefit from hormonal therapy in combination with treatment with anti-HER2 antibody Trastuzumab (Herceptin), depending on expression of HER2 or not [32, 33]. Finally, basal-like/triple negative breast cancer (TNBC) is a subtype with poor prognosis, due to lack of specific drug targets [31]. Chemotherapy is the primary treatment for this subtype [34].

Table 1. Molecular subtypes of breast cancer.

Subtype	Biomarkers	Therapy	Percentage
Luminal A	**ER+, PR+/-, HER2- and Ki67 low**	**Hormonal therapy**	**30-70**
Luminal B	**ER+, PR+/-, HER2+/- and Ki67 high**	**Hormonal therapy Trastuzumab**	**10-20**
HER2-enriched	**ER-, PR- and HER2+**	**Trastuzumab Chemotherapy**	**15-20**
Triple negative/ Basal-like	**ER-, PR- and HER2-**	**Chemotherapy**	**5-15**

1.2.5.2 TNM staging system

The TNM staging system declared by the American Joint Committee on Cancer (AJCC) is based on anatomical properties of the tumor as described in detail in table 2 [35]. TNM classification uses a combination of tumor size (T), lymph node involvement (N) and presence or absence of metastasis (M) [36]. This classification system provides a basis for survival prediction (prognosis), choice of initial therapeutic approaches and evaluation of therapeutic results [36].

Table 2. Breast cancer staging (summarized from AJCC). T1 = Tumor ≤2cm in greatest dimension, T2 = Tumor >2cm but ≤5cm, T3 = Tumor>5cm, N0 = No lymph node involvement, N1mi = Micrometastases less than 2mm, N1 = Metastases in 1-3 axillary lymph nodes, N2 = Metastases in 4-9 axillary lymph nodes N3 = Metastases in 10 or more axillary lymph nodes, M0 = absence of distant metastasis, M1 = Distant metastasis.

TNM staging system			
Stage 0	**T1**	**N0**	**M0**
Stage IA	**T1**	**N0**	**M0**
Stage IB	**T1**	**N1mi**	**M0**
Stage IIA	**T1**	**N1**	**M0**
	T2	**N0**	**M0**
Stage IIB	**T2**	**N1**	**M0**
	T3	**N0**	**M0**
Stage IIIA	**T1**	**N2**	**M0**
	T2	**N2**	**M0**
	T3	**N1**	**M0**
	T3	**N2**	**M0**
Stage IIIB	**T4**	**N0**	**M0**
	T4	**N1**	**M0**
	T4	**N2**	**M0**
Stage IIIC	**Any T**	**N3**	**M0**
Stage IV	**Any T**	**Any N**	**M1**

1.2.5.3 Tumor grade

Tumor grade classifies tumor tissues based on abnormality of the tumor cells microscopically [37]. It is used as a prognostic indicator of how quickly a tumor will grow and spread [37, 38]. Tumor grade represents the potential aggressiveness of a tumor taking into consideration the glandular/tubular formation, nuclear pleomorphism (variability in the size and shape of nuclei and nucleoli) and mitotic (cell division) count [39]. This classifies the tumor into three different grades, G1, G2 and G3. G1 represents low grade and well differentiated, G2 represents moderately differentiated and G3 indicates high grade and poorly differentiated [39].

1.2.6 Estrogen signaling pathway

Hormones often play a key role in breast cancer development [40, 41], modulating the structure and growth of epithelial tumor cells [40-42]. Estrogen is a steroid hormone, which plays essential roles in the regulation of growth and differentiation of the reproductive system [43, 44]. The major transcriptional effects of estrogens, which are nucleus-initiated, are mediated through a direct interaction with two estrogen receptors (ERs), ERα and ERβ [45]. Both receptors, which belong to the nuclear hormone receptor superfamily [46], are nuclear ligand-activated transcription factors that regulate the expression of specific sets of genes [44, 46]. It is well established that nuclear receptors contain several distinct domains, including the Activator Function 1 (AF1) domain at the N-terminus, the DNA Binding Domain (DBD), the Ligand Binding Domain (LBD), and the Activator Function 2 (AF2) domain at the C-terminus [47]. ERα and ERβ represent distinct gene products, but display high homologies in their LBDs and DBDs of 96% and 60%, respectively [48, 49]. ERs have both genomic (transcriptional) and non-genomic functions (Figure 3) [50]. Ligand unbound ERs are located in the cell membrane and cytoplasm [43]. Upon activation by estrogen, cytoplasmic ER homo- or hetero- dimerizes with regard to ERα and ERβ, and translocates into the nucleus, where it subsequently interacts with DNA either directly through specific hormone response regions located in or near promoter regions of target genes, or indirectly through other transcription factors, such as activator protein-1 (AP-1) and specific protein-1 (SP-1) [43, 51, 52]. In addition, ERs may regulate the expression of target genes through a ligand-independent manner in which ER directly or indirectly interacts with DNA subsequent to phosphorylation by some protein kinases, including MAP kinase [53]. Additionally, membrane-localized ER can stimulate a rapid response to estrogen through a non-genomic function in which the activation of the PI3K/MAPK signaling pathway stimulates proliferation and cell survival [43, 54].

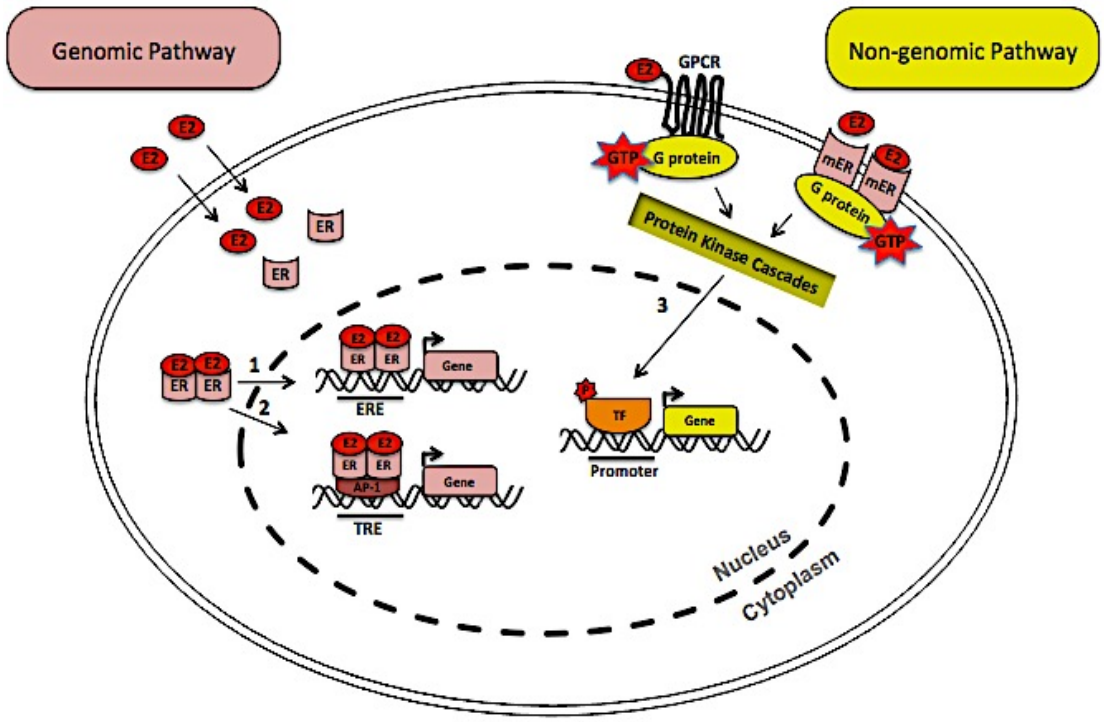

Figure 3. Estrogen signaling pathways. Estrogens induce gene regulation through different pathways including genomic and non-genomic pathways. In the genomic pathway, ERs can directly (classic, pathway 1) or indirectly (non-classic, pathway 2) bind to response elements, EREs or e.g. TREs. In the non-genomic pathway, membrane-bound ER (mER) stimulates a rapid response to estrogen through the activation of PI3K/MAPK signaling (Pathway 3). E2: Estradiol, ER: Estrogen receptor, GPCR: G protein complex receptor, TF: Transcription factor, TRE: TPA response element.

1.2.6.1 Estrogen receptor alpha (ERα) and breast cancer

An observation, over a hundred years ago, of the inhibitory effect of oophorectomy on patients having metastatic breast carcinomas provided the first evidence about the relationship between estrogens and breast cancer [55]. However, evidence that directly indicates that estrogens regulate breast cancer development was first provided in the 1950s with the discovery of estrogen receptor alpha (ERα) [56, 57]. At present, it has been well documented that over two-third of breast cancers overexpress ERα and nearby 70% of these respond to anti-estrogen therapy including Selective Estrogen Receptor Modulators (SERMs) and aromatase inhibitors (AIs) [58]. The unique feature of SERMs is tissue selective activity. There is supporting evidence that their activity is mainly determined by recruiting different cofactors (co-activators and co-repressors) to ER target genes in different types of cells and tissues [59, 60]. For example, tamoxifen and raloxifene antagonize estrogen in some tissues

of the body including breast tissue, while they may function like estrogen in other tissues. In contrast to raloxifene, tamoxifen has a similar structure as E2 and acts like estrogen in the uterus and therefore can increase the risk of endometrial cancer and uterine sarcoma [61, 62]. In addition, both tamoxifen and raloxifene can increase the risk of thrombosis [63]. Since in post-menopausal patients estrogen is mainly synthesized in peripheral tissues by the aromatase enzyme, aromatase inhibitors (AIs) such as anastrozole are clinically used for these patients [64]. Despite the advances of endocrine therapy in breast cancer, a significant percentage of ERα-positive tumors develop resistance. Therefore, investigation of other signaling pathways such as AP-1 and NF*k*B that interact with ERα signaling pathways is of interest as a means to find new therapeutic strategies to inhibit estrogen signaling in breast cancer.

1.2.6.2 Activator protein-1 (AP-1) and its association with breast cancer

AP-1 is a dimeric transcription factor complex that is composed of dimer combinations of four main family members including FOS, JUN, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families [65-67]. Although two family members including FOS (*c-Fos*, *Fra-1*, *Fra-2* and *Fos-B* genes, Figure 4A) and JUN (*c-Jun*, *Jun-B* and *Jun-D* genes, Figure 4B) have been best studied in breast cancer, further studies are required to understand the role of ATF and MAF protein families in breast cancer. Unlike the JUN family proteins, the FOS family proteins cannot homodimerize among themselves due to a small difference in amino acid composition in their leucine zipper regions [68]. However, they can dimerize with JUN proteins to generate JUN-FOS heterodimers. It has been reported that heterodimer complexes are more stable complexes than JUN-JUN homodimers, and bind strongly to response elements on DNA consisting of TPA response elements (TREs: 5'-TGAC/GTCA) in the promoter and/or enhancer regions of target genes (Figure 4D) [65, 66, 69-72]. In addition, JUN and FOS proteins can dimerize with other bZIP family proteins such as ATF and MAF family members. These heterodimers tend to bind to cyclic AMP-response elements (CREs) [73]. However, JUN-JUN and JUN-FOS dimers have a low affinity to CREs compared with TREs [74]. Studies show that JUN and FOS family members are overexpressed in different types of tumors including breast tumors and therefore could play an important role in tumorigenesis [75, 76]. While c-Jun is known as a positive regulatory factor of cell proliferation in breast cancer, Jun-B and Jun-D appear to play an opposite role having tumor suppressor ability [77, 78]. Additionally, some studies indicate that c-Fos, Fra-1, Fra-2 might be involved in invasion in breast cancer [65, 79]. Furthermore,

Fra-1 can influence cell proliferation by increasing cyclin D1, invasion by increasing MMP1 and MMP9, and angiogenesis through regulating the angiogenic factor VEGF [80, 81].

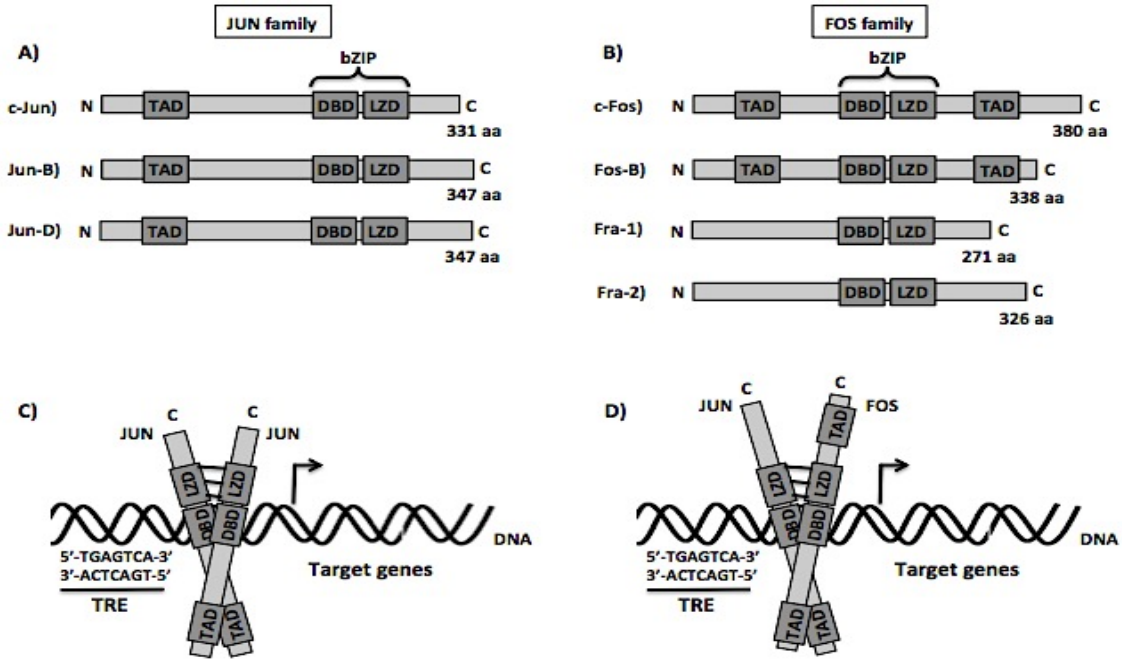

Figure 4. Schematic views showing AP-1 protein structures and dimerization properties. A and B) Simplified structures show the domains of JUN family and FOS family proteins. The bZIP contains the DNA binding domain (DBD) and the leucine zipper domain (LZD). The JUN proteins contain the transactivation domain (TAD) at their N-terminus regions, while the FOS proteins, except Fra-1 and Fra-2, contain TADs at both N- terminus and C-terminus regions. C and D). The LZD mediates protein dimerization necessary for DNA binding.

1.2.7 RBCK1, RNF31 and SHARPIN in breast cancer

RBCK1 (also known as HOIL1) is a 510 amino-acid protein. RBCK1 has been implicated in modulating cell cycle progression via regulation of TP53 activation of P21 transcription. RBCK1 also promotes proliferation by positively regulating ERα transcriptional activity and enhancing promoter binding, resulting in up regulation of down stream genes including *cyclin D*, *c-Myc* and *TFF1* genes [82]. In addition, it has been demonstrated that RBCK1 acts as a co-factor for ERα in ERα regulating its own expression [83]. Additionally, elevated mRNA expression of RBCK1 has been correlated with a less aggressive tumor phenotype [84]. It is important to note that in addition to an independent role of RBCK1 in diseases, RBCK1 is one of the components of a 600-kDa ternary protein complex known as linear ubiquitin chain assembly complex (LUBAC) [85]. The other components are RNF31 and SHARPIN [85]. LUBAC has been demonstrated to act as a positive regulator of the NF-*k*B pathway [86].

Interestingly, activated NF-*k*B pathway has been shown to stimulate proliferation and blocking programmed cell death (apoptosis) in human breast cancer [87].

The *RNF31* (also known as *HOIP* or *ZIBRA*) gene was first cloned from breast cancer cells based on its overexpression relative to normal breast cell lines [88]. *RNF31* encodes a 120-kDa protein that mainly localizes in the cytoplasm [89]. The protein contains a RING finger domain that has been reported to be involved in protein-DNA and protein-protein interactions [90-92]. RNF31 has E3 ubiquitin-protein ligase activity [93] and plays an important role in the ubiquitination pathways [93]. We have recently reported that RNF31 can stabilize the ERα protein through a monoubiquitination mechanism, and facilitate ERα-dependent proliferation of breast cancer cells [89].

SHARPIN was originally identified as a Shank-1binding protein in the post-synaptic density [94]. The physiological functions of SHARPIN have been best studied in chronic proliferative dermatitis, where spontaneous SHARPIN-deficient mice displayed severe chronic inflammatory skin lesions [95]. In addition, systematic analysis of 17 studies of Oncomine datasets revealed that SHARPIN mRNA expression levels are significantly elevated in invasive ductal breast carcinomas compared to non-tumor breast tissues and that this difference can differentiate breast tumors from non-tumor tissues (Area Under the Curve = 0.83) [96].

1.2.8 Fusion genes and their potential applications in breast cancer

A gene includes coding regions (exons) and non-coding regions (introns). Eukaryotic genes are clearly well defined in the genome. Genes are physically separated from each other through intergenic, noncoding regions (Figure 5). Genes can encode functional RNAs (non-coding RNAs such as transfer RNA and ribosomal RNA) or mRNAs (protein products). Transcription initiates from a transcriptional start site, which is governed by the promoter, and terminates at the regulated termination site of given gene [97]. However, in 1960, Peter Nowell and David Hungerford in Philadelphia detected an abnormal chromosomal marker in patients having chronic myeloid leukemia (CML), that was named the Philadelphia chromosome [98]. Thirteen years later, Janet Rowley found that the Philadelphia chromosome forms because of a translocation between chromosome 9 and 22 in patients with CML [99]. Later, several scientists showed that this translocation resulted in generating a new *BCR/ABL* fusion gene, encoding an abnormal fusion protein inducing CML [100-104].

At present, it is known that the formation of fusion genes is a common oncogenic mechanism found in various neoplasms including epithelial neoplasms [105]. Tumor-specific phenotypes can be determined by expression of such fusion transcripts [105]. In addition to fusion genes generated by chromosomal translocation (genomic rearrangements), fusion transcripts generated by *cis*- or *trans*-splicing of mRNA have also been identified in cancers including breast cancer (Figure 5) [106, 107]. Sequencing cDNA clone libraries and performing RNA-seq can detect such fusion transcripts [107, 108]. Such fusion mRNAs may encode novel proteins and alter cellular phenotypes [109]. One particularly frequent type of fusion transcripts that arise from two adjacent genes in the same coding orientation, is in the literature described as chimeric mRNAs, read-through fusion genes, co-transcription of adjacent genes coupled with intergenic splicing (CoTIS), transcription-induced chimeras, or conjoined genes [110-114]. For example, read-through fusion SCNN1A/TNFRF1A and CTSD/IFITM10 transcripts form through *cis*-splicing of pre-mRNA of two adjacent genes [108]. These were first detected in breast cancer cell lines, confirmed in breast cancer primary tumors, but have not been identified in normal breast tissues [108]. Reducing the expression of CTSD/IFITM10 using custom siRNA targeting decreased expression of the fusion mRNA and also decreased breast cancer cell proliferation [108].

Therefore, chimeric mRNAs and corresponding protein products might play crucial roles in tumorigenesis and can potentially become diagnostic and therapeutic targets. The identification of novel fusion genes and fusion mRNAs can open new opportunities for diagnosis and prognosis and finding new therapies.

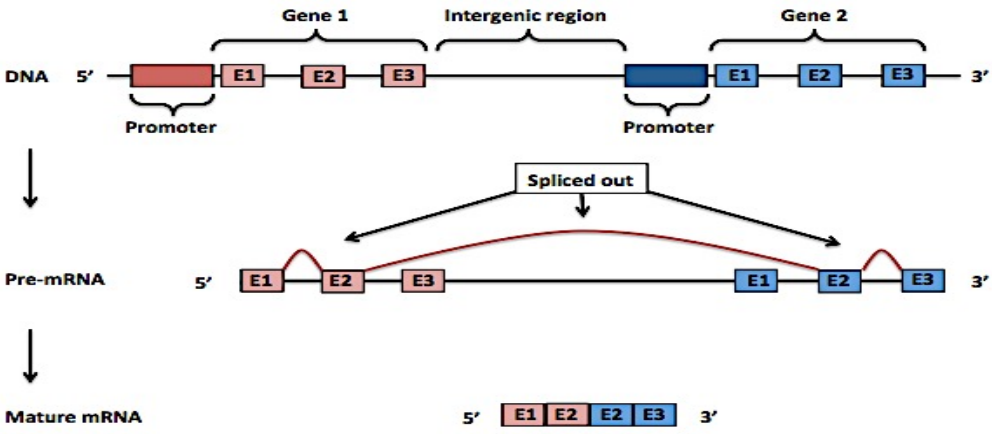

Figure 5. A schematic model for transcription-induced chimerism. Transcription spans both successive genes. When the pre-mRNA is spliced out, it involves a 5' splice site at the upstream gene and a 3' splice site at the downstream gene, with splicing of the intergenic region from the mature chimeric mRNA. The fusion transcript includes exons from both involved genes. Black lines represent introns (non-coding regions) and small boxes with E letters indicate exons (coding regions).

2 AIMS OF THE THESIS

General aim:

The overall aim of this thesis was to explore potential novel biomarkers for the use in breast cancer diagnosis as well as to improve therapeutic strategies.

Specific aims:

- To investigate the AP-1 family members as potential novel biomarkers in breast cancer.
- To further investigate the mechanism of action of RNF31, a modulator for ER signaling in breast cancer cells.
- To investigate the three members of the LUBAC protein complex as potential novel biomarkers in breast cancer.
- To investigate a potential fusion transcript and its corresponding protein in breast cancer.

3 MATERIAL AND METHOD

The details of the materials and methods are described in each study, while overall limitations and considarations are discussed in this section.

3.1 Clinical samples

For papers I, II, III, was analyzed breast tumor and adjacent non-tumor materials donated by female patients to the National Tumor Bank of the Cancer Institute of Iran. The specimens were collected upon surgery of the primary tumor, fresh frozen, and fixed in formalin and then kept at -80°C. Written informed consent was obtained from all patients who donated samples to the tumor bank. The biological material was transferred to Karolinska Institutet, Sweden under Material Transfer Agreement (MTA). A total of 72 primary breast tumors and 37 adjacent non-tumor tissues, for which 36 cases paired samples were available, were analyzed. Patients ranged from 24 to 85 years old with mean age 48.6 years and median age 46.5 years. All tumors were histologically defined as invasive ductal or lobular carcinomas. Receptor status including estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) was determined using Immunohistochemistry (IHC) assays, and was found to be positive in 47, 35 and 14 cases, respectively.

3.2 Ethical consideration

The National Research Ethics Committee of I.R of Iran and the Regional Research Ethics Committee of Karolinska Institutet approved the studies (approval numbers: 1-K90.P52 and 2012/774-31/2, respectively).

3.3 Cell lines

Cell lines are valuable sources for scientific studies. In this thesis MCF-7 and T-47D cell lines were used as representative models for ERα-positive breast cancer and MDA-MB-231 and BT-549 cell lines as models for triple-negative breast cancer (TNBC). Cell lines can be infinitely cultured and easily grown, and can be manipulated to undergo genotypic changes or phenotypic changes. In paper IV, we chose to focus on MCF-7 cells due to high expression of the *RNF31* gene. This cell line originates from work at the Michigan Cancer Foundation. The MCF-7 cell line was derived from a metastatic site in pleural effusion, following breast cancer mastectomy of a 69-year old Caucasian woman in 1970. Genetically, MCF-7 was described as having a karyotype with 88 chromosomes [115]. Depending on the condition of maintenance; today's cell lines range from 66 to 88 chromosomes [116]. Therefore, the

number of the chromosomes and culture conditions could explain controversial results reported from different laboratories.

3.4 EXPERIMENTAL TECHNIQUES

3.4.1 Small interference RNA transfection

Transient transfection of cells with small interfering RNA (siRNA) is a technique to investigate the role of one specific transcript and its corresponding protein. It is a posttranscriptional regulation process, where the siRNAs play a role as mediator for RNA interference. Generally, an enzyme called Dicer cleaves siRNAs from double-stranded RNA molecules. The resulting RNAs are incorporated into the RNA-induced silencing complex (RISC) in the cytoplasm, and silence the target mRNA via hybridization and its subsequent degradation. The main challenge for this method is off-target effects, which are related to the siRNA itself and most often arise from partial complementarity to non-specific mRNAs. These effects are mostly concentration dependent and therefore to minimize these effects, it has been recommended to reduce the siRNA concentration by performing a titration to find the optimal concentration.

3.4.2 Quantitative polymerase chain reaction

Real-time reverse transcription polymerase chain reaction (qRT-PCR) is a highly sensitive technique to quantify gene expression. In the TaqMan approach, which was used in our studies, the detection of PCR products is based on the addition of a gene specific probe having a reporter labeled with a fluorochrome at the 5' end and a quencher at the 3' end. When the probe is intact, the proximity of the reporter and the quencher dye allows the quencher to silence the fluorescence signal of the reporter via fluorescence-resonance energy transfer. When Taq DNA polymerase extends the strand from the 3' end of the primer; the exonuclease activity of the enzyme breaks down the probe, leading to release the reporter fluorescence. This process is repeated each cycle dependent on the speed of accumulation of released fluorescence, which will relate to the amount of starting material.

The quality of the initial template is the most important determinant in the biological relevance of conclusions derived from PCR. Therefore, we analyzed the RNA integrity by means of an Agilent 2100 system. After loading the RNA-containing sample on the Agilent chip, sample components are electrophoretically separated, generating an RNA integrity number (RIN) that provides an indication of RNA quality. In our studies, we used only samples with high-enough RIN values (RIN value ≥ 7) for subsequent RT-PCR. In order to choose the housekeeping gene to normalize the samples, we analyzed the expression of 16

candidate Endogenous Control genes using the TaqMan Endogenous Control Assay for 16 samples, including 11 tumors and 5 adjacent non-tumor tissues. Since the ubiquitin C (UBC) was the most stable housekeeping gene across the samples, we therefore chose this gene as an internal control.

3.4.3 Northern blot

Northern blot assay is a technique that is used to detect a particular RNA. This method uses electrophoresis to separate RNA by size. Detection is through a radioisotope labeled probe, which hybridizes complementarily to part or the entire target sequence. The advantages of this method include the detection of RNA size and high specificity that reduces false positive results. However, the method includes risk exposure to e.g. formaldehyde, radioactive material and UV light that should be always taken into consideration

3.4.4 Immunofluorescence assay

Immunofluorescence assay (IFA) is a technique that uses fluorescent dyes to identify the existence of antibodies bound to specific antigens [117]. IFA is a relatively simple and inexpensive method, which takes the advantages that the real morphology and subcellular protein expression localization can be evaluated [117]. For paper IV, we assessed the subcellular expression of RNF31 in tumor and adjacent non-tumor tissues. However, the reliability of IFA is highly dependent on the specificity of the antibody and the quality of the samples.

3.5 Statistical analysis

The normality of the continuous variables including relative mRNA expression levels was first examined by quartile-quartile plot (Q-Q plot). A Q-Q plot is a plot by which the quintiles of the data set are compared with normal theoretical quintiles. Since the data set did not meet the normal distribution (Figure 6A), the natural log (Ln) was applied for those continuous variables (Figure 6B) to be able to use the parametric statistical methods. However, the non-parametric methods could be applied when continuous variable does not meet normal distribution. Receiving Operating Characteristic (ROC) curve is a graphical method, to demonstrate the implementation of a binary classifier method to discern the true-positive values from false-positive values. One of the applications of this method in medicine is to evaluate diagnostic tests [118]. In our studies we used this to summarize and represent the discrimination between tumor and adjacent non-tumor tissues. In addition, student's t-test was used for comparing a continuous variable with a binary parameter such as ERα status, and one-way ANOVA was also used for comparing a continuous variable with several

categorical explanatory variables such as subtypes of breast cancer. Statistical analysis was calculated by means of SPSS statistical software and R software. Two-sided p-values of < 0.05 were considered to be statistically significant.

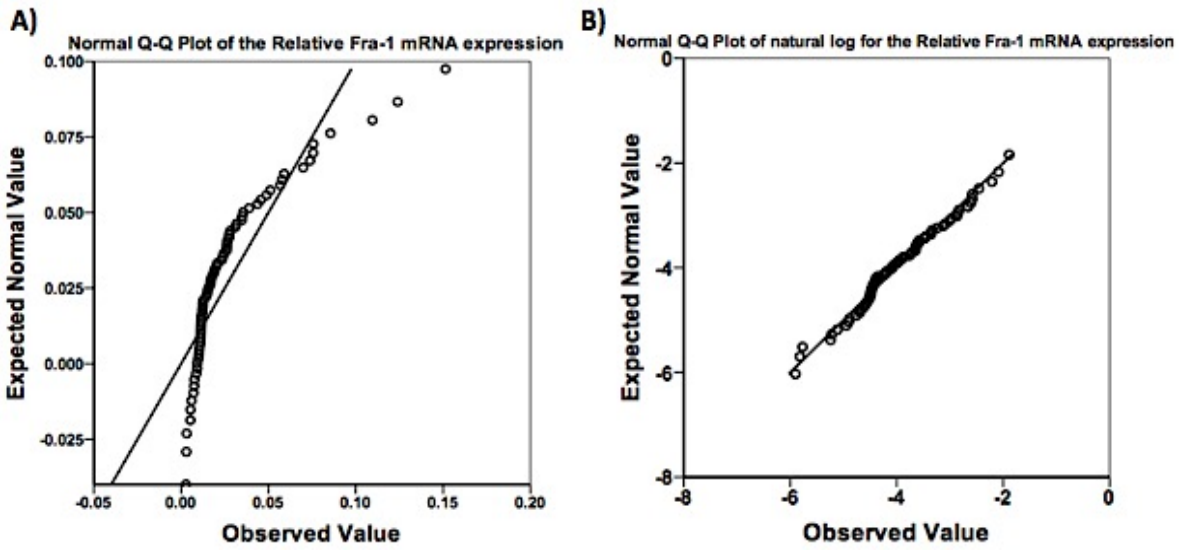

Figure 6. Applying a natural logarithm in the analyses when the data do not meet normal distribution. A) A Q-Q plot was applied to check the normality of the gene expression data. B) Natural logarithm (Ln) was applied for those data that did not meet the normality.

4 RESULTS AND DISCUSSION

4.1 STUDY I: Expression of activator protein-1 (AP-1) family members in breast cancer

AP-1 family members belong to the class of basic leucine zipper (bZIP) transcriptional factors [119]. They can bind to target genes' promoters through specific DNA sequences including TPA response elements (TREs) and cyclic AMP response elements (CREs) dependent on its composition. For example, JUN-JUN homodimers or JUN-FOS heterodimers bind to TREs on the promoters of target genes and then transactivate or repress transcription of the downstream target genes such as *EGFR*, *cyclin D*, *p53*, *p16*, *VEGF* and *MMPs* [119-122]. Depending on the activated or repressed genes, they can be involved in the cellular processes such as proliferation, apoptosis, differentiation or invasion [119-122]. A vast range of internal or external stimuli including hormones, growth factors, activated oncogenes, bacterial or viral infections and UV-radiation can induce AP-1 activity [119, 123]. Several studies have shown that either overexpression or lack of expression of some AP-1 genes can lead to oncogenic transformation, tumor growth promotion and tumor cell invasion [124, 125].

The purpose of this study was to investigate the expression of AP-1 family members in breast tumors and to correlate this expression with established prognostic biomarkers such as ERα, PR and HER2 status and also the TNM staging system and grade. For this, we employed real-time PCR on samples from 72 clinical human breast tumors and 37 adjacent non-tumor tissues. In addition, we assessed the protein expression levels using Western blot assay in a subset of breast tumors. Our findings showed that Fra-1, Fra-2, Jun-B and Jun-D mRNA levels were significantly up-regulated in tumors compared to adjacent non-tumor tissues ($p < 0.001$), proposing an oncogenic role for these genes in breast cancer. In addition, we observed that c-Fos and c-Jun mRNA levels were significantly down-regulated in tumors compared with adjacent non-tumor tissues ($p < 0.001$). In addition, Jun-B overexpression demonstrated an excellent discrimination ability to distinguish tumor tissues from adjacent non-tumor tissues as determined by ROC curve analysis, proposing it as a potential diagnostic biomarker for breast cancer. Moreover, Fra-1 was significantly overexpressed in triple-negative breast cancers (TNBC) compared to luminal tumors ($p = 0.01$). Since TNBC in comparison with other breast cancer subtypes has yet no specific therapeutic target, Fra-1 might be a potential therapeutic candidate for this subtype.

Some limitations in this study and also in the "*study III* " should be taken into consideration. In these studies tumor tissues are compared with adjacent tissues, ideally it would be better to compare with normal breast tissues if available. Tumor tissues might influence gene expression in the adjacent tissues. Secondly, an approach to investigate protein expression levels in all tumor samples and adjacent tissues would be preferred if possible. The adjacent non-tumor breast tissue is fatty therefore extracting the protein and running on the gel will be difficult. Third, the numbers of tumors belonging to the luminal B subtype was low why we analyzed luminal A and B together. Forth, since the numbers of tumors having stage IV was also low we merged stages III and IV together.

4.2 STUDY II: The atypical ubiquitin ligase RNF31 stabilizes estrogen receptor α and modulates estrogen-stimulated breast cancer cell proliferation

ERα is overexpressed in the majority of breast cancers and promotes estrogen-dependent cancer progression by regulating the expression of genes associated with cell proliferation. ERα status clinically is an important parameter as anti-estrogens or aromatase inhibitors can successfully treat ERα-positive breast cancers. Therefore, insights into the molecular mechanisms that regulate ERα expression and stability could be of highest importance to improve breast cancer therapy.

RNF31 is a RING (Really Interesting New Gene) finger protein containing a RING finger domain [126]. Most proteins that contain a RING finger domain can interact with DNA or other proteins [90-92]. In addition, they can play an important role in the ubiquitination pathways [93]. They can bind to ubiquitination enzymes and their substrate and act as ligases [93, 127]. RNF31 is highly expressed in heart, muscle and testis and is preferentially localized into the cytoplasm [128].

To investigate a potential role of RNF31 in proliferation of breast cancer cells, we depleted RNF31 in MCF-7 cells using siRNAs and found that this significantly reduced cell proliferation in an estrogen-dependent manner, mimicking the effect of ERα knockdown in this cell line model. The effect on proliferation and cell cycle arrest after RNF31 depletion could be rescued by ERα overexpression, showing that RNF31 can facilitate estrogen-dependent cell proliferation. In addition, RNF31 depletion could reduce ERα regulated reporter gene expression and also decrease the expression of endogenous ERα target genes such as *cyclin D1*, *ADORA1* and *TFF1*. Additionally, our results revealed that RNF31 co-localizes with ERα mainly in the cytoplasm and stabilizes ERα protein through mono-ubiquitination. Furthermore, analysis of RNF31 mRNA expression in breast cancer samples revealed that RNF31 is significantly higher expressed in breast tumor tissue compared to adjacent non-tumor tissues ($p < 0.001$). Overall, these findings propose RNF31 as a potential diagnostic and also as a therapeutic candidate target in ERα-positive breast cancer.

4.3 STUDY III: Expression of the three components of linear ubiquitin chain assembly complex in breast cancer

RBCK1, RNF31 and SHARPIN are three components of linear ubiquitin chain assembly complex, LUBAC, with an estimated molecular weight of 600-kDa. This complex induces activation of N*F*KB through conjugation of linear polyubiquitin chains to the NEMO (essential modulator for N*F*KB) subunit of the IKK complex [95, 129, 130]. RNF31 interacts with RBCK1 through interaction of the ubiquitin-associated (UBA) domain of RNF31 with the ubiquitin-like (UBL) domain of RBCK1, where this interaction seems to be essential to form the LUBAC complex [131]. It has been reported that two single nucleotide polymorphisms (SNPs) in the UBA domain of RNF31 lead to a stronger interaction with RBCK1 and increase LUBAC complex activity, causing cancer through activated N*F*KB pathway [132]. In study III, we aimed to examine the mRNA and protein expression levels of the three components of the LUBAC complex separately and correlate these with well-established markers in breast cancer. Thus, we employed real-time PCR assays on 72 human breast tumors and 37 adjacent non-tumor tissues. In addition, we evaluated protein expression levels using Western blot assay. We found that RBCK1 and SHARPIN, similar to RNF31 (paper II), were highly expressed in the 72 tumors compared to the 37 adjacent non-tumor tissues ($p < 0.001$). In addition, pairwise correlations of the expression of the three LUBAC components indicated a perfect correlation among non-tumor tissues, whereas the correlations decreased or disappeared among the tumor samples. This finding could be due to the accumulation of genomic mutations and tumor cellular environments, leading to up-regulation of genes in different types of cancer [133, 134]. In addition, the mRNA expression levels of these three genes differentiated tumor samples from non-tumor tissues as analyzed by ROC Curve analysis, proposing them as potential diagnostic markers in breast cancer. Additionally, we observed that RNF31 protein expression was significantly higher in ERα-negative tumors compared to ERα-positive tumors ($p = 0.034$), proposing an ERα-independent role for RNF31 protein involving other biological pathways. However, further studies are needed to understand the role of RNF31 in ERα-negative tumors. It is important to note that, when analyzing and interpreting these results, the earlier mentioned limitations for "*study I"* should be taken into account.

4.4 STUDY IV: Identification of a potential novel fusion transcript, RNF31/IRF9, in breast cancer

Since its discovery in 1960, knowledge regarding fusion genes has increased. Fusion genes can be formed through DNA rearrangement such as translocation, inversion, insertion or deletion of a part from chromosomes or without DNA rearrangement such as *cis*- or *trans*-splicing of pre-mRNAs [106, 107, 135]. The fusion gene may result in a new protein with altered properties and/or altered cellular localization [136]. Fusion gene production is principally considered as tumor-specific and therefore can have a therapeutic value in cancer treatment. For example, the *BCR/ABL1* fusion gene, a constitutively activated tyrosine kinase, is present in tumors cells of 95% of patients suffering from chronic myeloid leukemia [137] and inhibitors of this fusion protein has drastically changed the treatment outcome of CML [138].

When using a RNF31 antibody in Western blot assay, an unexpected protein band was detected in samples from the breast cancer cell line, MCF-7. As RNF31 has an estimated molecular weight of 120 kDa, a fusion protein containing RNF31 could be present in breast cancer cells. Further experiments with MCF-7 cells revealed that this fusion protein was enriched in the nuclei of the cells. Browsing on the genome showed that RNF31 and its neighboring gene, IRF9 are located in the same chromosomal orientation (Figure 7). We therefore hypothesized that the detected fusion protein might be a result of a fusion gene formed via forming a read-through of these two genes. In support of our hypothesis, it is known that fusion mRNAs can be formed by genes that are physically located up to 48 kb from each other (the median distance between two fused genes is 8.5kb) [139].

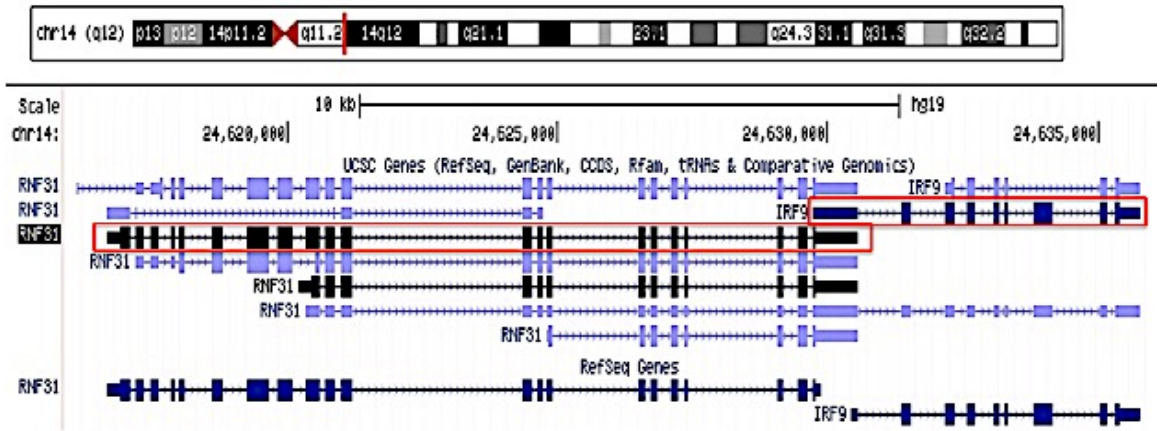

Figure 7. Genomic orientations of *RNF31* and *IRF9* genes. Figure demonstrates a zoomed-in view of *RNF31* and *IRF9* gene loci on chromosome 14.

The existence of this possible fusion protein was further studied in clinical breast tumors with Western blot analysis and immunofluorescence assays. This indicated that this fusion protein was not detected in adjacent non-tumor breast tissue. In order to detect the existence of a potential fusion transcript and also specify the potential transcript size, we performed Northern blot analysis. Our results showed the existence of a possible fusion transcript. However, the detected size of this transcript did not match the size of possible fusion protein and therefore further experiments are required to find out the possible reasons and full length of the potential fusion transcript.

5 CONCLUDING REMARKS

5.1 AP-1 IN TNBC

Triple-negative breast cancer (TNBC) accounts for around 15% of breast cancers and is the subtype that has the most pessimistic prognosis (133). Treatment of TNBC involves a combination of surgery (breast conserving or mastectomy), radiotherapy if breast-conserving surgery was done, and chemotherapy.

Our finding showing that Fra-1 is significantly overexpressed in TNBC compared to luminal carcinomas could have a therapeutic value for this subtype. However, more studies are needed in order to understand Fra-1's role and mechanism of action during initiation and/or development of established tumor.

5.2 RNF31 IN BREAST CANCER

RNF31 has been shown to possess enzymatic activity as an E3 ubiquitin ligase [128, 140]. However, the role of RNF31 in cancer especially breast cancer has been poorly investigated. Elevated expression of RNF31 in breast cancer [88] could be an indication proposing a potential biological function. Therefore, more studies on the potential function of RNF31 in breast cancer seem to be necessary.

Our findings revealing that RNF31 stabilizes ERα through mono-ubiquitination in ERα-positive tumors could propose a potential role for RNF31 in breast cancer and that it could have a potential therapeutic value in breast cancer. In addition, data showing a higher expression of RNF31 protein in ERα-negative tumors could propose an ERα-independent role involving other biological pathways. Therefore, more studies seem to be required to find out its role in ERα-negative tumors.

5.3 FUSION GENES IN BREAST CANCER

Fusion genes are chimeric products generated through fusion of previously separated genes with aberrant functions [105]. The produced proteins may present with abnormal expression levels, localizations and functions, causing abnormal cell proliferation and cancer development [141]. Therefore, tumor-specific phenotypes can be determined by expression of such fusion transcripts and corresponding proteins [105].

Our findings suggest a potential RNF31/IRF9 fusion transcript and corresponding protein. This fusion protein is expressed and enriched in the nuclei of breast tumors, while it is absent in normal breast tissues. Therefore, it can be a potential diagnostic and therapeutic candidate for breast cancer.

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